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FireMaster BP-6: Fractionation, Metabolic and Enzyme Induction Studies

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FireMaster BP-6 is a commercial polybrominated biphenyl (PBB) preparation containing a complex mixture of isomers with the major component being identified as 2,2',4,4',5,5'-hexabromobiphenyl. Column chromatographic techniques have been developed in which the crude FireMaster is separated into three fractions, F1, F2, and F3, in increasing order of polarity. F1 consists of highly purified 2,2',4,4',5,5'-hexabromobiphenyl (94%) whereas F2-F3 contain less of this isomer and correspondingly more of the other bromobiphenyl components. Previously we have shown that crude FireMaster BP-6 is metabolized in mammals to give hydroxylated degradation products and the metabolism of F1, not unexpectedly, gives comparable results.

It is well known that PBBs are effective inducers of diverse microsomal enzymes including the aryl hydrocarbon hydroxylase (AHH) system. The effects of FireMaster BP-6 and F1-F3 as AHH inducers have been investigated by using the following approach: the substrates used to monitor AHH activity are model halogenated aromatic compounds; the levels of metabolites and metabolite conjugates formed have been quantitated for control and induced enzymes; the levels of macromolecular adducts have also been quantitated for the inducers. This approach thus not only measures the rate of increase of detoxification products (metabolites and metabolite conjugates) but also monitors the macromolecule adduct formation which represents a toxification route. The effects of the PBBs as AHH inducers will be discussed in terms of the above approach.

The accidental contamination of animal feeds with a polybrominated biphenyl (PBB) flame retardant, FireMaster BP-6, has resulted in the widespread contamination of animals and humans in the state of Michigan. Since the ultimate identification of the toxic agent a number of papers have appeared in which the analysis, chemistry, photochemistry, metabolism and toxicology of brominated biphenyls has been described and much of this information has recently been summarized (*1*). FireMaster BP-6 has an average of six bromine atoms (by weight) per biphenyl and has been shown to be a mixture of several isomeric components with the major constituent having been identified as 2,2',4,4',5,5'-hexabromobiphenyl (*1*, *2*).

The biological properties of PBB suggest a marked similarity with those reported for the structurally related polychlorinated biphenyls (PCB) (*1*, *3*). The metabolism of selected brominated biphenyl isomers was also similar to the metabolism of the corresponding chlorinated biphenyl analogs (*4–6*). The metabolism of 2, 3, and 4-bromobiphenyl in the rabbit gave a series of hydroxylated products and for the latter isomer these were identified as 4'-bromo-4-biphenylol and 4'-bromo-3,4-biphenyldiol. The metabolism of corresponding deuterated metabolites (61 and 19% retention of deuterium, respectively) and the observed 1,2-migration of deuterium from the site of hydroxylation to the adjacent carbon atom (NIH shift) was consistent with the intermediacy of an arene oxide as shown in Eq. (1). The metabolism of 4,4'-dibromobiphenyl in the rabbit and pig gave similar results, in which the 1,2-migration of Br was observed. This experiment has been repeated on a young calf (*7*) and the same metabolites isolated from the rabbit, namely 4'-bromo-4-biphenylol, 3,4'-dibromo-4-biphenylol, and 4,4'-dibromo-3-biphenylol, were also identified as calf urinary metabolites. The metabolism of commercial FireMaster BP-6 gave a hydroxylated pentabromobiphenyl metabolite (*5*), and this product may have been formed by hydroxylation of the minor pentabromobiphenyl isomers or by hydroxylation/

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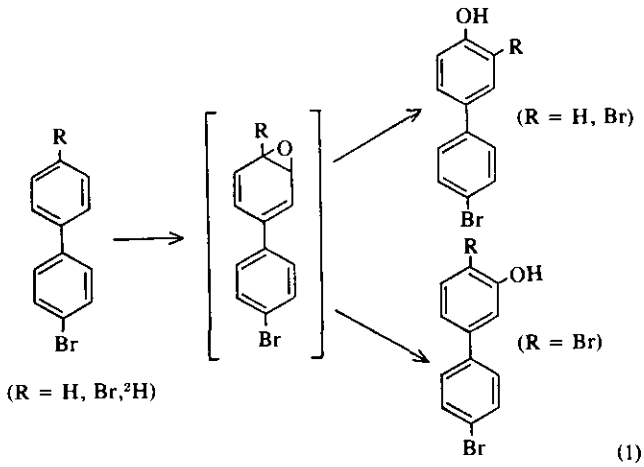
debromination of the hexabromobiphenyl components of the commercial mixture. This latter metabolic route has previously been reported for the metabolism of 2,2′-4,4′,5,5′-hexachlorobiphenyl PCB isomer (*8*). The limited metabolic data suggests that the higher brominated PBBs are metabolized at a much slower rate than the more metabolically active lower brominated isomers, and this also parallels the situation observed for the PCB isomers (*8*).



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Experimental Procedures

Fractionation of Commercial FireMaster BP-6

Commercial FireMaster (2.0 g) was applied to the top of neutral alumina column (145 g) and eluted with petroleum ether (bp 30–40°C). The eluate was continually monitored by gas chromatographic analysis (GLC) and divided into two major fractions, F_1 (least polar, 0.8 g, 750 ml) and F_2 (more polar, 0.4 g, 500 ml). The column was then eluted with diethyl ether (700 ml) to give the final polar fraction F_3 (0.5 g). A summary of the GLC analysis of FireMaster BP-6, F_1, F_2, and F_3 is given in Figure 1 and Table 1.

Metabolism of 2,2′,4,4′,5,5′-Hexabromobiphenyl in the Rat

The purified isomer, 2,2′,4,4′,5,5′-exabromobiphenyl (400 mg) as isolated above was dissolved in corn oil (10 ml) and equally administered by intraperitoneal injection to 8 male Wistar rats housed in metabolic cages. Urine and feces were collected for 20 days after administration of the chemical, and urinary and fecal metabolites were

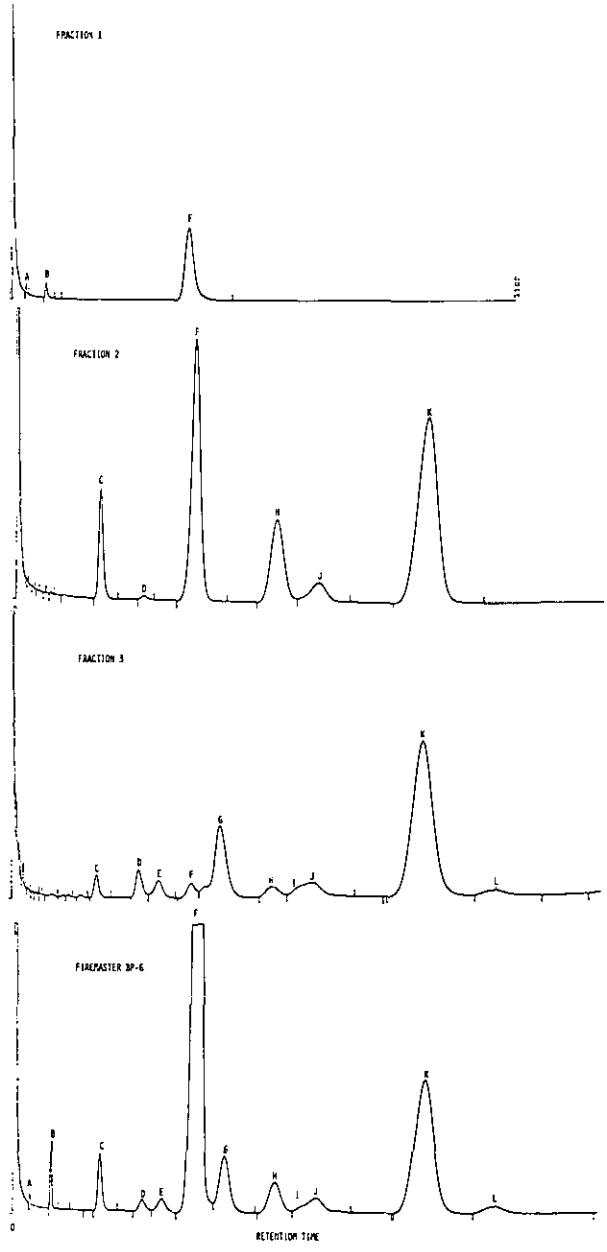


FIGURE 1. GLC analysis of FireMaster BP-6 and fractions 1, 2, and 3.

isolated and identified by a combination of thin-layer chromatographic and mass spectrometric procedures as described previously (*4–6*).

Microsomal Enzyme Induction Studies

The chemical inducers, FireMaster BP-6, F_1, F_2, and F_3 were dissolved in corn oil (100 mg in 2 ml) and administered to 63 day-old male Wistar rats by

Table 1. Composition of FireMaster BP-6 and fractions 1–3 obtained by alumina column chromatographic separation.

Peak[a]	Composition, %			
	FireMaster BP-6	Fraction 1	Fraction 2	Fraction 3
A	Trace	1.5		
B	1.0	3.5		
C	2.1		6.2	2.1
D	0.7		0.3	3.4
E	0.9			2.7
F[b]	61.3	95.0	30.7	2.6
G	5.4			17.6
H	3.3		13.3	2.8
I	1.1			2.9
J	1.5		4.2	3.8
K	22.7		45.0	60.7
L	0.7			1.2

[a] Determined by GLC separation on 1.5% OV-1 at 245°C.
[b] Identified as 2,2',4,4',5,5'-hexabromobiphenyl.

intraperitoneal injection. The inducer was administered on days 1 and 3 and the rats were sacrificed after 6 days by decapitation; the livers were quickly removed and cooled to 4°C. The microsomal enzyme fraction was prepared as described previously (*12*) with the following modifications. The 0.25*M* sucrose solutions were supplemented with 0.1*mM* EDTA; the 10,000*g* supernatant fraction was centrifuged at 100,000*g* for 90 min at 4°C on a sucrose cushion (4 ml, 1.6*M*); the microsomal band was then resuspended in sucrose–EDTA (8 ml, 0.25*M*, 0.1 mmole) to give a concentration of 1 g of wet liver per 2 ml of sucrose–EDTA solution. The resulting microsomal fraction contained 20–25 mg protein/ml. Cytochrome P-450 was determined as described previously (*13*). The microsome preparation was used immediately and incubated with [^{3}H]-4-chlorobiphenyl (0.1 μmole, 8 mCi/mmole) in dimethyl sulfoxide–Tween 80–ethanol (0.1 ml, 1:0.1:1) prepared by palladium-catalyzed exchange in trifluoroacetic acid (New England Nuclear). The incubation medium consisted of Tris hydrochloride buffer (50 μmole, pH 8.0), NADPH (1 μmole), and the microsomal preparation (1.0 ml) made up to a final volume of 3 ml with distilled water.

The mixture was incubated for 1 hr at 37°C and quenched by the addition of acetate buffer (3.0 ml, pH 4.7). Lipophilic metabolites were isolated by repeated extraction of the aqueous mixture with diethyl ether (4 × 6 ml) until only background radioactivity was present in the ether phase. The combined ether extracts were concentrated, redissolved in 1 ml of benzene and a 100 μl portion of this extract was resolved into three fractions by hin-layer chromatography (TLC) on silica gel HF_{254} (Merck) by use of chloroform–acetic acid (99:1) as the solvent. The three fractions corresponded to 4-chlorobiphenyl, 4'-chloro-4-biphenylol and 4'-chloro-3,4-biphenyldiol and these were removed from the TLC plate and counted directly in toluene based scintillation fluid. Trichloroacetic acid (TCA, 10%, 6 ml) was then added to the aqueous incubation mixture to precipitate the cellular macromolecules. After centrifugation the macromolecular pellet was washed with a second portion of trichloroacetic acid (6 ml). The combined TCA washes were diluted with distilled water to give a final volume of 25 ml, and an aliquot (1.0 ml) was counted in liquid scintillation cocktail (toluene–Triton X-100–2,5-diphenyloxazole, 1.0:06:0.007). The macromolecular pellet was washed repeatedly with hot methanol (4 × 6 ml) until only background counts were present in the final washing. The combined methanol washings were brought to a final volume of 25 ml and a 1-ml aliquot was counted in a toluene-based scintillation fluid. The macromolecular pellet was dissolved in NCS tissue solubilizer (1.0 ml, Amersham-Searle) for 2 hr at 50°C and an aliquot (200 μl) was counted in toluene-based liquid scintillation fluid to which glacial acetic acid (ca. 1–2 drops) was added to reduce the mixture to a pH of 5. Calibration curves and counting efficiencies were determined for all the counting fractions described above the corresponding dpm values were then derived.

For each enzyme inducer, three animals were used and three incubations from each animal were performed to give a total of nine runs/inducer. The counting results for these experiments were tabulated for all the microsomal subfractions and expressed as a percentage of the total recovered dpm.

Chromatographic and Spectroscopic Methods

The composition of the fractions was determined by GLC analysis on a Hewlett-Packard model 5710 chromatograph equipped with a flame ionization detector and operated at 245°C A 0.3 cm × 2 m glass column was packed with 1.5% OV-1 on Gas-Chrom Q (80–100 mesh). Operating conditions were: helium carrier gas flow, 30 ml/min; flame ionization and detector temperatures, 300°C; hydrogen flow, 60 ml/min; air flow, 200 ml/min. Mass spectra were recorded on a Varian MAT CH-7 or V6 Micromass 7070 mass spectrometer equipped with electrical detection.

Results and Discussion

The alumina column chromatographic procedure was successful in separating the commercial FireMaster BP-6 into three fractions with a variable content of the 2,2',4,4',5,5'-hexabromobiphenyl

isomeric component. Fraction 1 contained ca. 95% of the major isomer (peak F) with trace quantities of the lower PBB isomers (i.e., peaks A and B); FireMaster BP-6 contained 61.3% of this component, and F_2 and F_3 contained 30.7 and 2.6%, respectively, of 2,2',4,4',5,5'-hexabromobiphenyl. Fractions 2 and 3 were much richer in the heptabromobiphenyl component (peak K) as well as the other more polar isomers.

The metabolism of the purified 2,2',4,4',5,5'-hexabromobiphenyl did not yield any major quantity of metabolite although mass spectrometric analysis of the urine and fecal extracts did indicate trace quantities of hexabromobiphenyl metabolites had formed. The results were not surprising in light of the persistence and stability of the highly halogenated aromatics.

Table 2. Summary of the metabolism of [^{3}H]-4-chlorobiphenyl with control and induced hepatic microsomes.

Fraction (No.)	Total recovered activity, %				
	Control	F_1	F_2	F_3	FMBP6
Unreacted chlorobiphenyl (1)	80.0 ± 58.0	79.2 ± 40.0	49.8 ± 34.4	34.8 ± 31.4	36.6 ± 24.8
4'-Chloro-4-biphenylol (2)	12.0 ± 2.53	9.00 ± 2.60	28.1 ± 3.5	38.0 ± 10.2	38.6 ± 7.3
4'-Chloro-3,4-biphenyldiol (3)	1.25 ± 0.29	2.25 ± 0.59	3.85 ± 0.78	4.12 ± 0.96	3.75 ± 0.27
Low molecular weight 4-chlorobiphenyl conjugates (4)	5.34 ± 1.00	7.47 ± 1.53	14.8 ± 1.3	18.1 ± 2.8	16.5 ± 0.34
Methanol washes (5)	0.96 ± 0.14	1.49 ± 0.22	2.03 ± 0.06	1.64 ± 0.08	1.71 ± 0.64
Macromolecular-4-chlorobiphenyl adducts (6)	0.428 ± 0.036	0.564 ± 0.085	1.34 ± 0.27	3.31 ± 0.91	2.88 ± 0.502

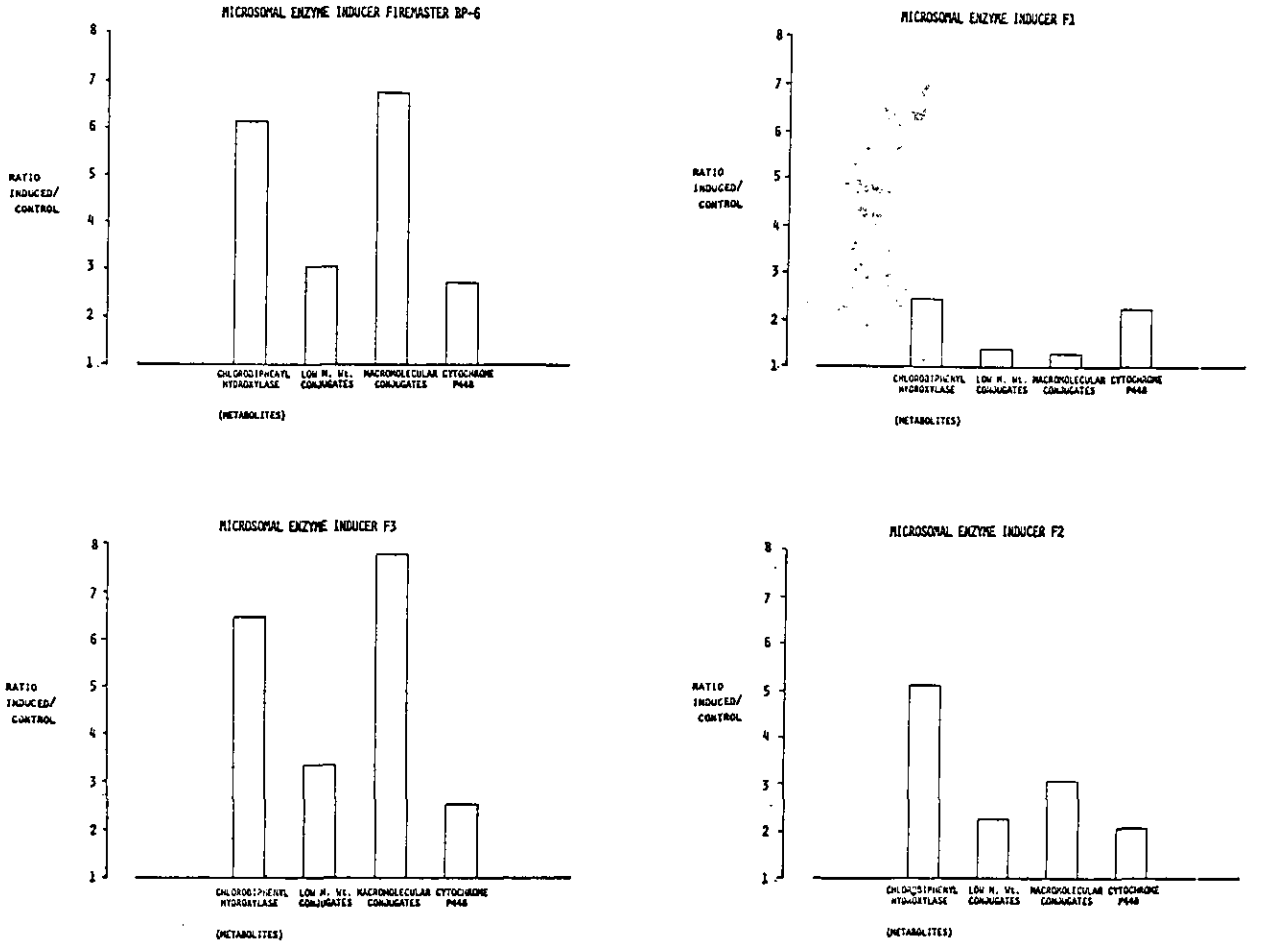


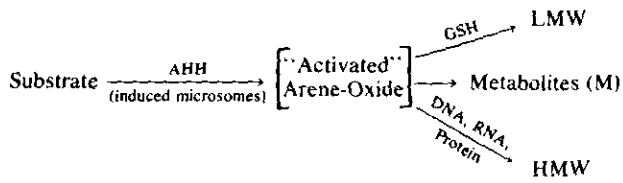
FIGURE 2. Effects of FireMaster BP-6 and fractions 1, 2, and 3 as microsomal enzyme inducers.

Previous research (*9*) has shown that FireMaster BP-6 is a highly effective inducer of microsomal enzymes, including cytochrome P-450, epoxide hydratase, ethoxycoumarin-*O*-deethylase, ethylmorphine-*N*-demethylase, aniline hydroxylase and AHH (using benzo[a]pyrene as the substrate). The induction of this latter enzyme activity was particularly striking, since values of 1500% of control were obtained 192 hr after administration of the inducer (150 mg/kg). At lower inducer concentrations (25 mg/kg), the AHH activity was still 730% of the control value 192 hours after injection of the chemical inducer.

The results obtained for FireMaster BP-6, F_1, F_2, and F_3 as chemical inducers with the use of [^{3}H]-4-chlorobiphenyl as the substrate are summarized in Table 2 and Figure 2. The overall increase in 4-chlorobiphenyl hydroxylase is variable with the smallest increase observed with F_1, ca. 250% of control, whereas the remaining fractions and FireMaster BP-6 gave values between 500-650% of control. Clearly the pure hexabromobiphenyl isomer (F_1) is a microsomal enzyme inducer, but the presence of the other isomeric components markedly enhances this activity. This result suggests that either one or more of the isomers present in F_2, F_3 and FireMaster BP-6 but not in F_1 is an extremely potent AHH inducer or that a synergistic effect exists and the multiplicity of isomers enhance the activity of the individual components. This synergism in enzyme induction is currently under investigation in our laboratory.

Although the percentage increase in AHH activity is reflected in the above data this does not give a complete picture of the cellular effects of this increased activity. It is known that the AHH enzyme system is not only involved in the metabolic process but the metabolically activated substrate intermediate can also react with low molecular weight nucleophiles such as glutathione to give low molecular weight adducts (LMW) and high molecular weight macromolecules such as protein, RNA, and DNA to give high molecular weight adducts (HMW). This latter fraction is associated with the carinogenic or toxic event since "it has become axiomatic that the induction of cancer by chemical carcinogens results from such covalent binding to one or more of these cellular macromolecules" (*14*). Thus a more complete assessment of the PBB inducers can be ascertained (*15*) by determining the incorporation of the radioactive substrate into the trichloroacetic acid low molecular weight conjugate fraction and the insoluble HMW fraction, and the results are shown in Table 2 and Figure 2. These data clearly indicate that the radioactivity incorporated into the HMW and LMW components was much lower for F_1 than the other three inducers, and this result parallels the induction of metabolite formation by these PBB fractions.

A summary of the AHH-mediated metabolism of the substrate is given in Eq. (2). Both the M and LMW fractions are more water-soluble forms of the



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original hydrocarbon which can be readily exreted. These have been classified as detoxification (D) products. The macromolecular adducts (HMW) have been labeled as toxification (T) products, and a T/D index has been defined (*15*):

$$T/D = 1000\,[HMW(M + LMW)]$$

Thus the T/D index can be derived for any chemical inducer and used to assess the potential toxicity mediated by the activation of the AHH enzyme system. The T/D value obtained for the control microsomes was 23, whereas values of 30, 29, 55, and 49 were obtained for F_1, F_2, F_3, and FireMaster BP-6, respectively. These values reflect a marked increase in the T/D index for all the PBB fractions but a proportionally higher increase for F_3 and FireMaster BP-6. Preliminary experiments with FireMaster BP-6 as an inducer and other aromatic substrates also result in a marked increase in the T/D index for the induced hepatic microsomes as compared to control values.

Currently research is in progress to explore the utility of this approach using an array of halogenated aromatic inducers and diverse hydrocarbon substrates.

REFERENCES

1. Kay, K. Polybrominated biphenyls (PBB) environmental contamination in Michigan. Environ. Res. 13: 74 (1977).
2. Sundstrom, G., Hutzinger, O., and Safe, S. Environmental chemistry of flame retardants Part II. Identification of jor component of the flame retardant, FireMaster BP-6. Chemosphere 5: 11 (1976).
3. Department of Health Education and Welfare. Final report of the subcommittee on the health effects of polychlorinated biphenyls and polybrominated biphenyls. Washington, D. C., July 1976.
4. Safe, S., Jones, D., and Hutzinger, O. The metabolism of 4,4′-dihalobiphenyls. J. Chem. Soc. 1976: 357.
5. Kohli, J., and Safe, S. The metabolism of brominated

aromatic compounds. Chemosphere 5: 443 (1976).
6. Kohli, J., et al. The metabolism of bromobiphenyls. Biochem. Pharmacol. in press.
7. Safe, S., and Chittim, B., unpublished results.
8. Sundstrom, G., Hutzinger, O., and Safe, S. The metabolism of chlorobiphenyls. Chemosphere 5: 267 (1976).
9. Dent, J. G., Netter, K. J., and Gibson, J. E. The induction of hepatic microsomal metabolism in rats following acute administration of a mixture of polybrominated biphenyls. Toxicol. Appl. Pharmacol. 38: 237 (1976).
10. Stonard, M. D. Mixed type hepatic microsomal enzyme induction by hexachlorobenzene. Biochem. Pharmacol. 24: 1959 (1975).
11. Alvares, A. P., Bickers, D. R., and Kappas, A. Polychlorinated biphenyls: a new type of inducer of cytochrome P-448 in the liver. Proc. Nat. Acad. Sci. (U.S.) 70: 1321 (1973).
12. Petropaolo, C., and Weinstein, I. B. Binding of [^{3}H]benzo[a]pyrene to natural and synthetic nucleic acids in a subcellular microsomal system. Cancer Res. 35: 2191 (1975).
13. Omura, T., and Sato, R. The carbon monoxide binding pigment of liver microsomes. J. Biol. Chem. 239: 2370 (1964).
14. Heidelberger, C. Chemical carcinogenesis. Ann. Rev. Biochem. 44: 79 (1975).
15. Crawford, A., and Safe, S. An assessment of the effects of enzyme inducers on aryl hydrocarbon hydroxylase activity. Res. Commun. Chem. Pathol. Pharmacol. 18: 59 (1977).